Cloning the *Dmrt1* and *DmrtA2* genes of ayu (*Plecoglossus altivelis*) and mapping their expression in adult, larval, and embryonic stages

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Abstract: The *Dmrt* family of genes are involved in sex differentiation in different species of invertebrates, and some vertebrates including human. In this study, we cloned the full-length cDNA of ayu (*Plecoglossus altivelis*) *Dmrt1* and *DmrtA2*. Sequence and phylogenetic tree analyses showed ayu *Dmrt1* showed highest similarity to that of *Oncorhynchus mykiss* while ayu *DmrtA2* is most similar to that of *Oryzias latipes*. Fluorescence-based quantitative reverse transcription PCR (qRT-PCR) revealed the *Dmrt1* was predominantly expressed in the testis. At the larval stages, *Dmrt1* mRNA expression level was highest during 52–64 days post hatching (dph) and at the gastrula stage during embryonic development. *DmrtA2*, meanwhile, was specifically expressed in the ovary and was highly expressed in the female brain tissue, but not male brain tissue. During the larval stages, *DmrtA2* expression remained high before day 34, and then fluctuated while generally decreasing. During embryonic development, *DmrtA2* expression increased gradually and peaked at the hatching stage. Our data suggest that ayu *Dmrt1* might participate in the differentiation and maintenance of testis while *DmrtA2* may play a role in ovary-differentiation and mature-ovary maintenance. *DmrtA2* might also participate in brain development.

Keywords: Ayu; *Dmrt*; Sequence analysis; Expression

The ayu (*Plecoglossus altivelis*; sweetfish) is a popular migratory fish and one of the most economically important freshwater fish in southeastern China and Japan due to both its perceived nutrition and taste. Belonging to the class Osteichthyes, suborder Salmonoidei, and family Plecoglossidae, the ayu is also well-known for its comparatively fast reproduction cycles. Paired with the consumer preference for the fish's superior flavor and faint smell, the ayu has accordingly been called the "the King of Freshwater Fish" in Asia. The name may be more appropriately titles "the Queen of Freshwater Fish," as the females reproduce more quickly and are more desirable for consumption, so much so that the sales price of female ayu is nearly double that of a male (Cao et al, 1981; Cao et al, 1982; Li et al, 1985; Wang et al, 1998).

Given the consumer preference for females and the rise in the fishes popularity in Asia, finding a way to improve the breeding production and develop selective breeding and female seeding techniques may prove worthwhile. Observational research on the wild and the cultured populations of ayu showed that the adult male and female ayu display a sexual size dimorphism, but for this study we wanted to take a more cohesive view of the underlying dimorphism. Accordingly, in this study analyzed the gonad transcriptome to identify the members of the sex-related double-sex and Mab-3-related transcription factor (*Dmrt*) gene family. The Dmrt family of transcription factors, including the sex-determinant (Dsx) of *Drosophila melanogaster*, and the Mab-3 of nematode, are characterized by a DNA-binding DM-domain, an unusual zinc-finger structure (Kim et al, 2003; Ren et al, 2001). Currently, at least 8 genes in the *Dmrt* family (*Dmrt1–Dmrt8*) (de Grandi et al, 2000; Guo et al, 2005; Kettlewell et al, 2000; Kondo et al, 2002; Nanda et al, 1999; Shibata et al, 2002) have

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been detected across many species, including mammals (de Grandi et al, 2000), reptiles (Kettlewell et al, 2000), birds (Nanda et al, 1999), fish (Kondo et al, 2002), and amphibians (Shibata et al, 2002). A previous study indicated that the *Dmrt* gene family plays a major role in an organism's sex development (Capriglione et al, 2010; de Grandi et al, 2000; Kettlewell et al, 2000; Marchand et al, 2000; Yang et al, 2013;), while another study pointed out that *Dmrt* participated in somite development (Seo et al, 2006).

In particular, *Dmrt1*, one of the prominent genes in this family, plays an important role in sex-determination and differentiation in *Drosophila melanogaster*, *Nematodes*, *Oryzias latipes*, and mice. To date, the regulatory mechanisms underlying sex determination and differentiation in some species has been studied. For example, knocking out *Dmrt1* expression during male differentiation leads to gonad feminization in chickens (Smith et al, 2009) while in mice, the *Dmrt1* gene inhibits female programming in the testis after birth (Matson et al, 2011). In some fish, *Dmrt1* shows testis-specific expression (Guan et al, 2000; Liu et al, 2004; Kobayashi et al, 2004; Shin et al, 2009) while in others it shows expression in both the ovary and testis, with the expression in the testis being marginally higher than that in the ovary (Guo et al, 2004; Marchand et al, 2000). In male fish, *Dmrt1* is generally expressed only in the sertoli cells, but the same is not true for females (Kobayashi et al, 2008). Meanwhile, *DmrtA2* (*Dmrt5*), another member of the *Dmrt* gene family, has been identified in some fish, including the zebrafish (Guo et al, 2004), swordfish (Veith et al, 2006), and rice field eel (Zhang et al, 2006). Previous studies on *DmrtA2* function mainly found relation to the brain and gonads; though in humans, the function is associated with development of the cerebral cortex (Saulnier et al, 2013) while in toads it is related to the formation of the olfactory placode nerve (Parlier et al, 2013).

Although many previous reports focused on the molecular heredity of ayu (Huang et al, 2004; Wang et al, 2011; Dong & Nobuhiko, 2003), they were restricted to the isoenzyme, biochemical heredity, and RAPD genetic diversity. To our knowledge, there are no previous studies focusing on ayu sex differentiation and its regulation. In this study, we screened the members of *Dmrt* gene family from the transcriptome and cloned *Dmrt1* and *DmrtA2*. We examined their expression in different adult tissues, and during larval and embryonic developmental stages in order to provide a better understanding of the relevant mechanism at play in the sex differentiation among ayu.

MATERIALS AND METHODS

Experimental materials and reagents

Ayu fish were obtained from Qingjiang base, Zhejiang Fisheries Research Laboratory. From August-October, different tissues were sampled were frozen, immediately dissected, and then stored in liquid nitrogen at −80℃ until further analysis. The embryos of different stages were collected at 1.5 mL DNase/RNase-free centrifuge tube immediately and then stored in liquid nitrogen.

TRIzol reagent, pMD19-T vector, PrimeScript RT reagent Kit, 3′-Full RACE, and 5′-Full RACE Kit were purchased from Takara company. Other reagents were domestic analytical reagents and were purchased from Bioequip. *Escherichia coli* DH5α cells were stored in our lab.

cDNA cloning and amino acid sequence analysis

The total RNA of different tissues was isolated using RNAiso Plus (Takara) according to the manufacturer's instructions. The RNA was digested by RNase-free DNase I and purified. The reverse transcription reaction was performed in a 10-μL volume using the PrimeScript RT reagent Kit (Takara). The reaction was performed at 37°C for 15 min and 85°C for 5 s, and then stored at −20°C.

Specific primers were designed for RACE and RT-PCR (Table 1) based on the partial transcriptome sequence, and *β-actin* was used as a control. All the primers were synthesized by Sangon Biotech.

Table 1 Primers for *Dmrt* cloning and analysis in ayu (*P. altivelis*)

Primer	Sequence (5′–3′)
D1-3-I	AGTCAGAGACCTTCACTGTGGATTC
D1-3-O	CATCAACTCCCTTGTCAACTCG
D1-5-I	GGACAGTCTTCCCACACACTCTAAT
D1-5-O	GCTCATTCTTCACCACAATCTCAG
D1-y-F	CCTCAGACCTGGTGGTGGATG
D1-y-R	GTTGGGAATCTGGTACTGCTGATAG
DA2-3-I	GCAGCCAAACTCACCTCAC
DA2-3-O	AGTCGAGGACTGGCTTTCAT
DA2-5-I	CAACGCTGACACGACACCG
DA2-5-O	CAACGCTGACACGACACCG
DA2-y-F	TGAAAGGCCACAAGCGTTATT
DA2-y-R	CGGGCTTCGTTCTCTTCCT
β-actin-F	TCGTGCGTGACATCAAGGAG
β-actin-R	CGCACTTCATGATGCTGTTG

All procedures were performed according to users' manuals (3′-Full RACE Core Set Ver. 2.0 and 5′-Full RACE Kit, Takara). The PCR products were separated on a 2% agarose gel, cloned into the pUC-19 vector, and sequenced.

All *Dmrt* protein sequences from different species were aligned using the NCBI blast program (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Phylogenetic trees were constructed using MEGA5.0 (Tamura et al, 2011) using the Neighbor-joining (NJ) method, yielding an unrooted consensus tree with a 1 000 bootstrap replicates.

Dmrt expression analysis at different stages by RT-PCR

To quantify *Dmrt* expression, we used RT-PCR according to the manufacturer's protocols. PCR cycling conditions were adjusted to the following parameters: 95°C for 30 s, then 60°C for 30 s, and finally 72°C for 30 s for 40 cycles for *Dmrt* and *β-actin* in a 20-L volume containing SYBR Green (TaKaRa). The primers for *Dmrt* and *β-actin* are listed in Table 1. All experiments were performed in triplicate to ensure concordance and eliminate potential errors. The resulting data were analyzed via the $2^{-\Delta\Delta Ct}$ method (Livak et al, 2001).

RESULTS

Cloning and sequence analysis of ayu *Dmrt*

The cloned 1684 bp *Dmrt1* transcript (GenBank accession number: KC899210) consisted of an 882-bp open reading frame (ORF) that coded 293 amino acids, a 674-bp 3′-untranslated region (UTR), and a 116-bp 5′-UTR. NCBI conserved domain search (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) showed that the DM-domain is represented by amino acids 27–73, and the Dmrt1 super-family conserved domain is represented by amino acids 187–246 (Figure 1).

We obtained the 2738-bp cDNA sequence of *DmrtA2* (Figure 1; GenBank accession number: KF296364), The ORF (1311 bp) coded 435 amino acids and contained the DM, DMA, and DMB conserved domains. The 3′-UTR (not including poly(A) signals) and 5′-UTR were 948 bp and 479 bp, respectively. NCBI conserved domain search (http://www.ncbi.nlm.nih.gov/Structure/ cdd/wrpsb.cgi) showed that the DM-domain included amino acids 51–96, and the DMA conserved domain included amino acids 187–246.

However, genomic analysis (data not shown) indicated that this transcript includes a 297-bp intron in its coding region, suggesting that it might represent an alternative splicing form of *DmrtA2*.

Similarity and phylogeny of amino acids of ayu *Dmrt*

Alignment analysis showed that the identity between Dmrt1 of ayu and other teleostean is greater than 60%, with the most similar species being the rainbow (81.5% identity) and the least similar being medaka (62% identity). The identity between the *Dmrt1* of Ayu and other vertebrates was less than 60% (Table 2). The NJ-phylogenetic tree showed that all the fish cluster into a subgroup, and the other vertebrates cluster into a separate group. In the fish subgroup, the fish from the Cypriniformes, Siluriformes, and Perciformes form a clade, and the ayu cluster with the rainbow trout (Figure 2). *DmrtA2* amino acid comparison also showed a similarity greater than 70% between ayu and other teleostean, with the highest similarity being medaka (84%). The similarity with the other vertebrates was less than 60% (Table 2). The NJ-phylogenetic tree for *DmrtA2* indicates that the ayu *DmrtA2* cluster with the other fish and stay farther away from humans and mice (Figure 2).

Dmrt gene expression in ayu adult tissue

In different adult tissues, *Dmrt1* showed the highest expression level in the testes and the lowest level in the intestine. Using the intestinal expression level as a reference, the expression levels in testis, ovary, and female spleen were 63.1, 3.2, and 1.6 times, respectively. *DmrtA2* showed the highest expression in the female brain and the lowest expression in the male brain. Using the expression level from the male brain as a reference, the expression levels in the ovary, female brain, and female intestine were 1.3, 37.6, and 2.5 times of that in the male brain, respectively (Figure 3).

Dmrt expression in ayu larvae

The development of ayu larvae is a slow process (Li et al, 1985), so we accordingly sampled tissue from 10 days post hatching (dph) to 70 dph. For *Dmrt1*, the stages showing highest expression levels occurred between 52 dph and 64 dph. Using the expression level at 40 dph as a baseline, the expression levels at 52 dph and 64 dph were 81.5 and 88.9 times that at 40 dph, respectively. For *DmrtA2*, the expression levels increased from 10 to 34 dph and then decreased to a minimum,

Dmrt1

DmrtA2

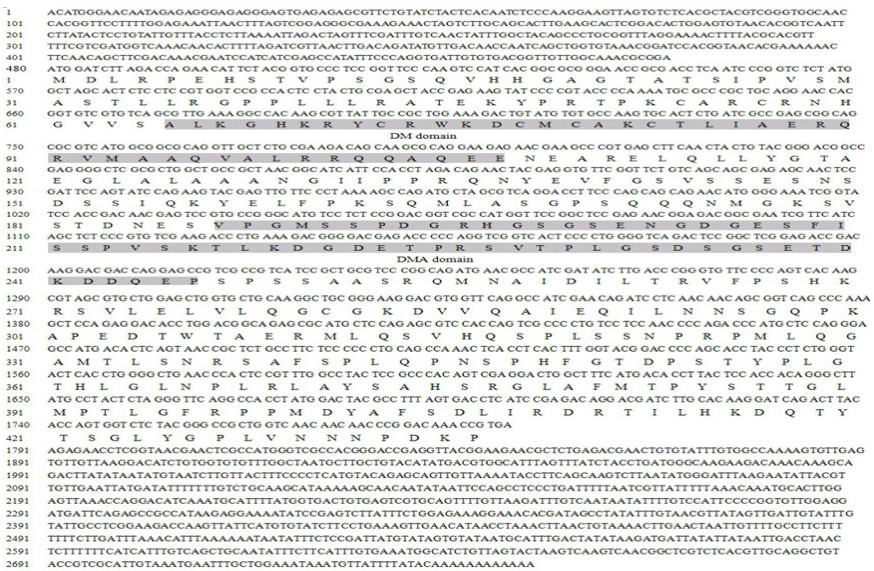

Figure 1 Sequences of ayu (*P. altivelis*) *Dmrt1* and *DmrtA2* cDNAs, codons, and amino acids

with the exception of the expression levels at 40 dph and 52 dph. *DmrtA2* expression reached its peak at 34 dph, which was 10.6 times that at 70 dph (Figure 4).

Expression of *Dmrt* genes in ayu embryos

In different embryonic developmental stages, ayu *Dmrt1* was expressed throughout embryonic development, reaching a peak at the gastrula stage, and a low at the cleavage state. Using the cleavage state as a reference, the expression levels during the morula, blastula, gastrula, neuronal, heartbeat, incubation, and earlier alevin stages were 41.5, 65.3, 250.2, 45, 78.5, 35.1 and 9.4 times, respectively. *DmrtA2* expression peaked at the earlier alevin stage, and the expression levels in the morula, blastula, gastrula, neural, heartbeat, incubation, and earlier alevin stages were 1.2, 1.4, 1.5, 7.7, 18 74.5 and 112.2 times of that in the cleavage stage, which was considered a reference (Figure 5).

DISCUSSION

Previous studies have established *Dmrt1* as a sex-determinant gene in a variety of species, and showed that its expression is sex-specific. For example, in the black porgy (Shin et al, 2009), *Tilapia mossambica* (Guan et al, 2000), and medaka (Kobayashi et al, 2008), *Dmrt1* is expressed only in the testis. Similar to these results, the *Dmrt1* expression in ayu testis was about 20 times of that in the ovary (Figure 3). However, *Dmrt1* not only shows

Table 2 Comparison of amino acid sequence identities of ayu Dmrt1 and DmrtA2

GenBank accession numbers	Species names	Gene names	Identity with ayu Dmrt1/DmrtA2（%）
AAG17544	*Oncorhynchus mykiss*	*Dmrt1*	81.50
ACR77511	*Clarias gariepinus*	*Dmrt1a*	78.50
HM245921	*Tachysurus fulvidraco*	*Dmrt1*	78
Q71MM5	*Danio rerio*	*Dmrt1*	73.50
ABK88911	*Paramisgurnus dabryanus*	*Dmrt1*	73
ABM54575	*Silurus meridionalis*	*Dmrt1b*	73
CAQ52797	*Dicentrarchus labrax*	*Dmrt1*	72
ABK15558	*Epinephelus coioides*	*Dmrt1*	68.50
AFA45126	*Gobiocypris rarus*	*Dmrt1*	68.50
BAM62886	*Parajulis poecilepterus*	*Dmrt1a*	62.20
ACD62373	*Epinephelus merra*	*Dmrt1a*	62.20
AAP84972	*Acanthopagrus schlegelii*	*Dmrt1*	64
BAC65996	*Oryzias curvinotus*	*Dmrt1*	62
Q9PTQ7	*Gallus gallus*	*Dmrt1*	58
AAD40474	*Homo sapiens*	*Dmrt1*	56
AAF12826	*Mus musculus*	*Dmrt1*	55.50
Q3LH63	*Xenopus laevis*	*Dmrt1*	54.50
Q76L87	*Oryzias latipes*	*Dmrt5*	84
Q5UU75	*Danio rerio*	*Dmrt5*	83
Q2I327	*Xiphophorus maculatus*	*Dmrt5*	83
ACU30591	*Monopterus albus*	*Dmrt5*	82
AFA46804	*Gadus morhua*	*Dmrt5*	81
NP_001033039	*Takifugu rubripes*	*DmrtA2*	81
Q6YHU8	*Oreochromis niloticus*	*Dmrt5*	80
AEM44777	*Xenopus tropicalis*	*Dmrt5*	72
AAN10254	*Mus musculus*	*Dmrt5*	57
AAI43801	*Homo sapiens*	*DmrtA2*	57

strong expression in the testis, but also a subtle expression in the ovary of zebrafish (Guo et al, 2004), rainbow trout (Marchand et al, 2000), and *Takifugu rubripes* (Shen et al, 2007). Ohmuro-Matsuyama et al (2003) found that *Dmrt1* is expressed in many different tissues of Medaka.In our study, we also detected low *Dmrt1* expression in ayu intestine and kidney, leading us to infer that *Dmrt1* not only plays an important role in testis maintenance but also may play some other yet unknown roles.

Compared to the adult tissues, *Dmrt1* expression occurred during the entire embryonic developmental stages in both *Pelteobagrus fulvidraco* (Li et al, 2012) and *Cynoglossus semilaevis* (Sun et al, 2008). Intriguingly, the expression levels different between the two fish in that the peak expression levels occurred at a different time. During this present study, we found similar results, with the expression peak at the gastrula stage. The previous studies showed that during larval development of *Pelteobagrus fulvidraco* (Li et al, 2012), *Dmrt1* was expressed from 1 to 51 dph and reached a peak at 31 dph, while another study (Sun et al, 2008) found that in *Cynoglossus semilaevis* the expression of *Dmrt1* at 22 dph was higher than during the other developmental stages. In our study, *Dmrt1* was expressed from 10 to 70 dph, reaching a peak at 64 dph. Moreover, examination of a paraffin section of ayu gonad revealed that the period from 40–60 dph was the key time for testis formation. During this time, *Dmrt1* expression was easily detectable, implying that *Dmrt1* plays an important role in the process of testis-differentiation.

DmrtA2 is mainly involved in the development of the brain and gonad. In mice embryos, for example, *DmrtA2* is expressed primarily in both the brain and the gonads. Moreover, expression in the female ovary is higher than that in the male testis, and very lowly

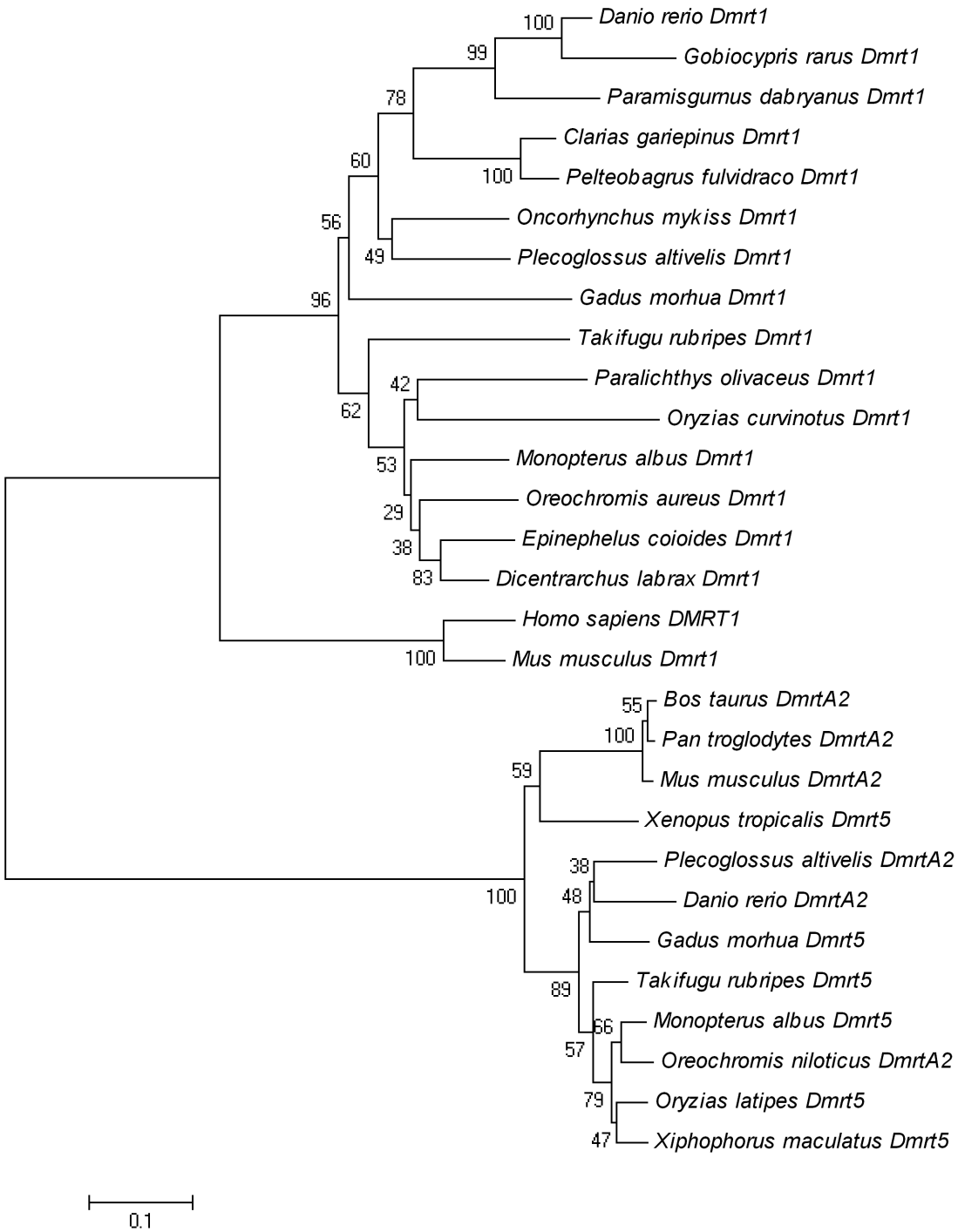

Figure 2 NJ-Phylogenetic tree based on *Dmrt1* and *DmrtA2* amino acid sequences

Numbers at the nodes denote the bootstrap values for 1000 replicates.

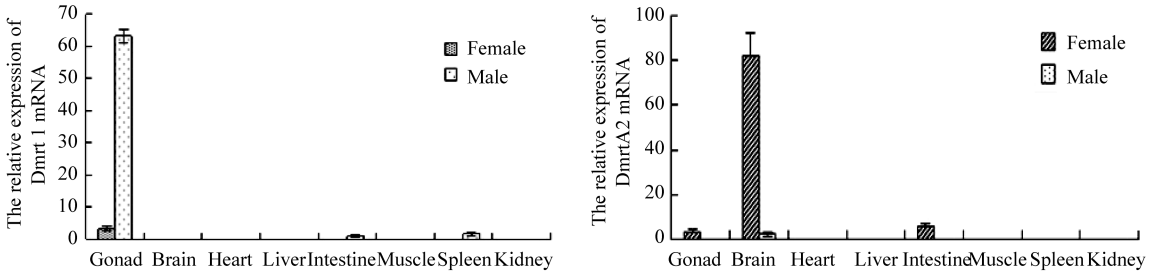

Figure 3 RT-PCR analysis of *Dmrt1* and *DmrtA2* transcripts in different ayu (*P. altivelis*) adult tissues

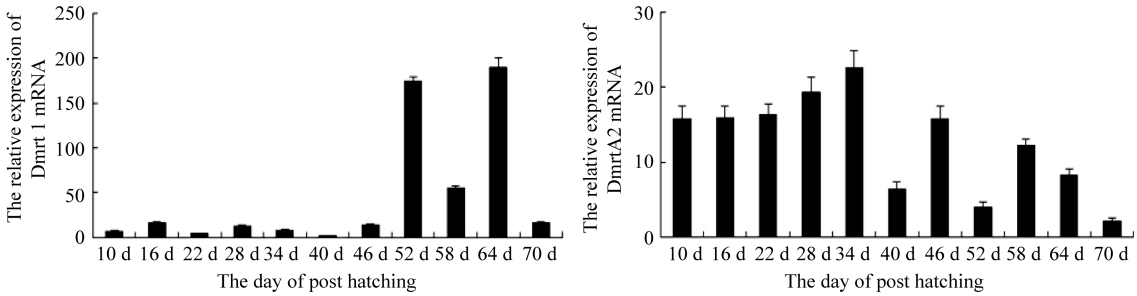

Figure 4 RT-PCR analysis of *Dmrt1* and *DmrtA2* transcripts of ayu (*P. altivelis*) at different post-hatching developmental stages

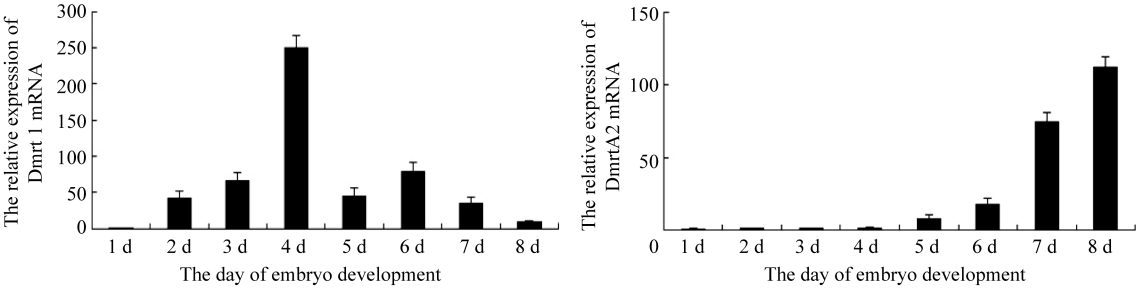

Figure 5 RT-PCR analysis of *Dmrt1* and *DmrtA2* transcripts of ayu (*P. altivelis*) at different embryonic stages

The numbers of the x-axis represent the following stages: 1 d, fertilized eggs; 2 d, morula stage; 3 d, blastula stage; 4 d, gastrula stage; 5 d, eye pouch period (neurula stage); 6 d, heartbeat period (tail-bud stage); 7 d, hatching stage; 8 d: early larva.

expressed in other tissues (Kim et al, 2003). In the puffer fish (Yamaguchi et al, 2006), *DmrtA2* was expressed in the brain and eye, with no expression in gonads, while in the swordfish (Veith et al, 2006) it was expressed in the olfactory placode and midbrain and in the zebrafish (Guo et al, 2004) it was expressed primarily in the midbrain of the embryo stage. Recently, Gennet et al (2011) also found that *DmrtA2* had an effect on the brain nerve in mice, a finding concordant with our results. It then stands to reason that *DmrtA2* may play a vital role in the development of the ayu brain. Guo et al (2004) had previously cloned the *DmrtA2* gene and found that it was expressed in the germ cells of zebrafish, mainly in the spermatogonia, spermatocytes, spermatoblasts, and oocytes, again implicating *DmrtA2* in gonad development. Zhang et al (2006) elaborated on this and found that *DmrtA2* was expressed in the gonad tissues of three types of rice field eels and that its expression in testis was higher than that in the ovary. And again, in the Pearlescent shellfish, *DmrtA2* participates in gonad development (Yu et al, 2009). In our experiment, we found that *DmrtA2* was expressed only in the ovary and had detectable expression before 34 dph, which coincides with the critical time for ovary differentiation. Considering these results, we propose that *DmrtA2* likely participates in the regulation of gonad development, and may do so across numerous fish species.

In addition to influencing sex determination, *Dmrt* genes may be involved in the regulation of tissue development. Ayu *Dmrt1* and *DmrtA2* exhibited different expression patterns in females and males, but both were also expressed in a variety of other tissues, suggesting that in ayu, the *Dmrt* genes likely play multiple roles in organ development. Further studies may help to reveal the roles of *Dmrt1* in testis development and *DmrtA2* in brain development.

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